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TRANSFORMANTS PRODUCING SECONDARY METABOLITES MODIFIED WITH FUNCTIONAL GROUPS, AND NOVEL BIOSYNTHESIS GENES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to transformants producing secondary metabolites modified by functional groups, more specifically, to transformants producing secondary metabolites in which a benzene ring is modified at the para-position with a functional group containing a nitrogen atom. Furthermore, the present invention relates to novel genes involved in a biosynthetic pathway from chorismic acid to p-aminophenylpyruvic acid.

2. Description of the Related Art

Since organisms produce a number of various kinds of secondary metabolites having biological activity, research for utilizing these metabolites for drugs for humans and animals, agricultural chemicals, and the like has been actively carried out. However, secondary metabolites from organisms can rarely be utilized for practical use as they are, and accordingly they are generally modified with various functional groups to optimize their biological activity. A modification with a functional group containing a nitrogen atom, such as a nitro group and amino group, is one of the most important modifications.

Chemical methods are available for modifying a certain substance with a nitro group. However, introduction of a nitro group into a benzene ring specifically at the para-position using a chemical method is extremely difficult, and its yield is very low. Furthermore, when a substance to be modified with a nitro group is as complex as a secondary metabolite from an organism, it is even more difficult to specifically modify a benzene ring at the para-position with a nitro group.

On the other hand, methods of introducing an amino group are generally classified into two groups, i.e., enzymatic methods and chemical methods. In enzymatic methods, an enzyme called aminotransferase (EC 2.6.1 group) is used. However, substances which can be a substrate for the aminotransferase are limited,

for producing a secondary metabolite having a benzene ring skeleton substituted at the para-position with a functional group containing a nitrogen atom, which comprises the steps of culturing the above-mentioned transformant and collecting the secondary metabolite having a benzene ring skeleton substituted at the para-position with a functional group containing a nitrogen atom.

Another objective of the present invention is to provide a novel gene involved in the biosynthetic pathway from chorismic acid to p-aminophenylpyruvic acid.

Novel genes according to the present invention are a gene encoding the amino acid sequence of SEQ ID NO: 2 or a modified sequence of SEQ ID NO: 2 having 4-amino-4-deoxychorismic acid synthase activity; a gene encoding the amino acid sequence of SEQ ID NO: 4 or a modified sequence of SEQ ID NO: 4 having 4-amino-4-deoxychorismic acid mutase activity; and a gene encoding the amino acid sequence of SEQ ID NO: 6 or a modified sequence of SEQ ID NO: 6 having 4-amino-4-deoxyprephenic acid dehydrogenase activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the restriction map of a DNA fragment isolated from Streptomyces venezuelae and the position of open reading frames thereon.

Figure 2 shows the construction of plasmid pTrc-papA.

Figure 3 shows the amino acid analyzer chromatograms used for detecting enzyme activity of a papA gene product.

Figure 4 shows the construction of plasmid pTrc-papB.

Figure 5 shows the amino acid analyzer chromatograms used for detecting enzyme activity of a papB gene product.

Figure 6 shows the construction of plasmid pET-papC1.

Figure 7 shows the amino acid analyzer chromatograms used for detecting enzyme activity of a papC gene product.

Figure 8 shows the construction of plasmids pPF260-A2 and pPF260-A3.

Figure 9 shows the restriction map of the 6-kb HindIII fragment containing the Abp1 gene.

Figure 10 shows the ~~construction and~~ restriction map of

pABPd.

Figure 11 shows the construction of plasmid pPF260-B3.

Figure 12 shows the construction of plasmid pPF260-C3.

Figure 13 shows the HPLC chromatograms used for detecting PF1022 derivatives in which a benzene ring is modified at the para-position with a nitro group or an amino group.

~~DETAILED~~ DESCRIPTION OF THE ~~INVENTION~~ PREFERRED EMBODIMENTS

Deposition of microorganisms

The strain PF1022 described in Example 5 was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, the Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba City, Ibaraki Prefecture, Japan), dated January 24, 1989. The accession number is FERM BP-2671.

The transformant 55-65 of Mycelia sterilia was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, the Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba City, Ibaraki Prefecture, Japan), dated September 17, 1999. The accession number is FERM BP-7255.

Escherichia coli (JM109) transformed with plasmid pUC118-papA was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, the Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba City, Ibaraki Prefecture, Japan), dated September 17, 1999. The accession number is FERM BP-7256.

Escherichia coli (JM109) transformed with plasmid pTrc-papB was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, the Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba City, Ibaraki Prefecture, Japan), dated September 17, 1999. The accession number is FERM BP-7257.

Escherichia coli (JM109) transformed with plasmid pET-papC was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, the Ministry of International Trade and Industry (1-3 Higashi

according to an ordinary method by appropriately selecting a medium, culture conditions, and the like.

The medium can be supplemented with a carbon source and nitrogen source that can be anabolized and utilized, respectively, by the transformant of the present invention, various vitamins, various amino acids such as glutamic acid and asparagine, trace nutrients such as nucleotides, and selective agents such as antibiotics. Further, organic and inorganic substances that help the growth of the transformant of the present invention or promote the production of the secondary metabolite of interest can be appropriately added. Further, if necessary, a synthetic medium or complex medium which appropriately contains other nutrients can be used.

Any kind of carbon source and nitrogen source can be used in the medium as long as they can be utilized by the transformant of the present invention. As the anabolizable carbon source, for example, various carbohydrates, such as sucrose, glucose, starch, ~~glycerin, glucose,~~ sucrose, glycerol, fructose, maltose, mannitol, xylose, galactose, ribose, dextrin, animal and plant oils and the like, or hydrolysates thereof, can be used. The preferable concentration generally is from 0.1% to 5% of the medium.

As the utilizable nitrogen source, for example, animal or plant components, or exudates or extracts thereof, such as peptone, meat extract, corn steep liquor, and defatted soybean powder, organic acid ammonium salts such as succinic acid ammonium salts and tartaric acid ammonium salts, urea, and other various inorganic or organic nitrogen-containing compounds can be used.

Further, as inorganic salts, for example, those which can produce sodium, potassium, calcium, magnesium, cobalt, chlorine, phosphoric acid, sulfuric acid, and other ions can be appropriately used.

Of course, any medium which contains other components, such as cells, exudates or extracts of microorganisms such as yeasts, and fine plant powders, can be appropriately used as long as they don't interfere with the growth of the transformant and the production and accumulation of the secondary metabolite of

solution. The entire ligated mixture was used to infect Escherichia coli LE392 strain using a Gigapack III Plus Packaging Kit (Stratagene) to form phage plaques. The 1.3×10^4 (2.6×10^4 PFU/ml) phage library obtained by this method was used for cloning of the Abp1 gene.

Cloning of the Abp1 gene from the genomic DNA derived from substance PF1022-producing microorganism

A probe to be used was prepared by amplifying the translation region of the Abp1 gene by the PCR method. The PCR was carried out using the genomic DNA prepared from the substance PF1022-producing microorganism as described above as a template and synthetic primers 8-73U and 8-73R, according to a LETS GO PCR kit (SAWADY Technology). The PCR reaction for amplification was conducted by repeating 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 90 seconds at 72°C. DNA sequences of the 8-73U and 8-73R are as follows:

8-73U: CTCAAACCAGGAACTCTTTC (SEQ ID NO: 15)

8-73R: GACATGTGGAAACCACATTTTG (SEQ ID NO: 16)

The PCR product thus obtained was labeled using an ECL Direct System (Amersham Pharmacia Biotech). The phage plaque prepared as described above was transferred to a Hybond N+ nylon transfer membrane (Amersham Pharmacia Biotech), and after alkaline denaturation, the membrane was washed with 5×SSC (SSC: 15 mM trisodium citrate, 150 mM sodium chloride) and dried to immobilize the DNA. According to the kit protocol, prehybridization (42°C) was carried out for 1 hour, after which the above-mentioned labeled probe was added, and hybridization was carried out at 42°C for 16 hours. ~~The probe~~ The nylon membrane was washed according to the kit protocol described above. ~~The~~ nylon membrane ~~with the washed probe~~ was immersed for one minute in a detection solution and then photosensitized on a medical X-ray film (Fuji Photo Film Co., Ltd.) to obtain one positive clone. Southern blot analysis of this clone showed that a HindIII fragment of at least 6 kb was identical with the restriction enzyme fragment long of the genomic DNA. Figure 9 shows the restriction map of this HindIII fragment. The HindIII fragment was subcloned into pUC119 to obtain pRQHin/119 for use of the following

experiment.

Construction of expression vector

The promoter region and the terminator region of the Abp1 gene were amplified by the PCR method using pRQHin/119 as a template. The PCR method was carried out using a PCR Super Mix High Fidelity (Lifetech Oriental Co., Ltd.) with primers ABP-Neco and ABP-Nbam for promoter amplification and ABP-Cbam and ABP-Cxba for terminator amplification. The amplification reaction was conducted by repeating 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 90 seconds at 72°C. The DNA sequences of ABP-Neco, ABP-Nbam, ABP-Cbam and ABP-Cxba are as follows:

ABP-Neco: GGGGAATTCGTGGGTGGTGATATCATGGC (SEQ ID NO: 17)

ABP-Nbam: GGGGGATCCTTGATGGGTTTTGGG (SEQ ID NO: 18)

ABP-Cbam: GGGGGATCCTAAACTCCCATCTATAGC (SEQ ID NO: 19)

ABP-Cxba: GGGTCTAGACGACTCATTGCAGTGAGTGG (SEQ ID NO: 20)

Each PCR product was purified with a Microspin S-400 column (Amersham Pharmacia Biotech) and precipitated with ethanol, after which the promoter was double-digested with EcoRI and BamHI, the terminator was double-digested with BamHI and XbaI, and the resulting fragments were ligated one by one to pBluescript II KS+ previously digested with the same enzymes. The product was digested with XbaI, and a destomycin resistance cassette derived from pMKD01 (WO 98/03667) was inserted to construct pABPd (Figure 10). pABPd has the promoter and terminator of the Abp1 gene.

An approximately 2 kbp BclI DNA fragment was prepared from plasmid pUC118-papA described in Example 2. This fragment was inserted into the BamHI site of the expression vector pABPd for PF1022-producing microorganism to obtain plasmid pPF260-A.

Next, pPF260-A was double-digested with restriction enzymes PstI and BamHI to prepare a DNA fragment of approximately 1.7 kbp. This fragment was subcloned into PstI and BamHI sites of pUC119 to obtain plasmid pUC119-A. Treatment for site-directed mutagenesis was carried out with pUC119-A as a template DNA and the oligonucleotide of SEQ ID NO: 21 as a primer using a Muta-Gene in vitro Mutagenesis Kit (Bio-Rad) to obtain plasmid pUC119-A1.

Next, pUC119-A1 and pPF260-A were double-digested with

restriction enzymes PstI and ~~BamHI~~ BamHI to prepare DNA fragments of approximately 1.7 kbp and approximately 8.6 kbp, and then these fragments were ligated to obtain plasmid pPF260-A2. Further, pPF260-A2 was digested with restriction enzyme XbaI and then self-ligated using T4 DNA ligase to obtain plasmid pPF260-A3.

Example 6: Construction of plasmid pPF260-B3 for introduction into PF1022-producing microorganism

Plasmid pPF260-B3 for expressing the papB gene in a PF1022-producing microorganism was constructed as shown in Figure 11.

An approximately 0.3 kbp BamHI DNA fragment was prepared from plasmid pTrc-papB described in Example 3. This fragment was inserted into the BamHI site of the expression vector pABPd (Example 5) to obtain plasmid pPF260-B. pPF260-B was digested with restriction enzyme XbaI and then self-ligated using T4 DNA ligase to obtain plasmid pPF260-B1.

Next, pPF260-B1 was digested with restriction enzyme PstI to prepare a DNA fragment of approximately 0.6 kbp. This fragment was subcloned into the PstI site of pUC118 in such a manner that the papB gene and the lacZ' gene aligned in the same direction to obtain plasmid pUC118-B. Treatment for site-directed mutagenesis was carried out with pUC118-B as a template DNA and the oligonucleotide of SEQ ID NO: 22 as a primer using a Muta-Gene in vitro Mutagenesis Kit (Bio-Rad) to obtain plasmid pUC118-B1.

Next, pUC118-B1 and pPF260-B1 were digested with restriction enzyme PstI to prepare DNA fragments of approximately 0.6 kbp and approximately 8.0 kbp, and then these fragments were ligated to obtain plasmid pPF260-B3.

Example 7: Construction of plasmid pPF260-C3 for introduction into PF1022-producing microorganism

Plasmid pPF260-C3 for expressing the papC gene in a PF1022-producing microorganism was constructed as shown in Figure 12.

An approximately 1 kbp BamHI DNA fragment was prepared from plasmid pET-papC described in Example 4. This fragment was inserted into the BamHI site of the expression vector pABPd

ABSTRACT OF THE DISCLOSURE

An objective of the present invention is to provide a transformant altered so as to produce a secondary metabolite in which a benzene ring of the secondary product is modified at the para-position with a functional group containing a nitrogen atom. A transformant according to the present invention is a transformant of an organism producing a secondary metabolite having a benzene ring skeleton without substitution at the para-position with a functional group containing a nitrogen atom, said transformant being transformed by introducing a gene involved in a biosynthetic pathway from chorismic acid to p-aminophenylpyruvic acid so as to produce a secondary metabolite having a benzene ring skeleton substituted at the para-position with a functional group containing a nitrogen atom. Another objective of the present invention is to provide a novel gene involved in the biosynthetic pathway from chorismic acid to p-aminophenylpyruvic acid. A novel gene according to the present invention comprises genes encoding the amino acid sequences of SEQ ID NOs: 2, 4 and 6 or modified sequences thereof.

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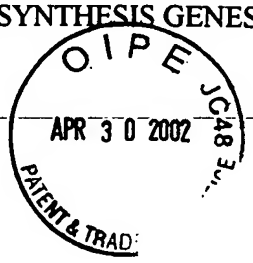

Receipt of the following papers is acknowledged:

1. Preliminary Amendment
2. Version With Markings To Show Changes Made
3. Sequence Listing
4. Diskette

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